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Inhibition of large T antigen ATPase activity as a potential strategy to develop anti-polyomavirus JC drugs

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Abstract

Introduction—This study evaluates polyomavirus JC (JCV) large T antigen (LTA) as a potential target for drug development. LTA is a hexameric protein with a helicase activity that is powered by ATP binding and hydrolysis. The helicase and ATPase function is critical for viral replication.

Methods—Recombinant JCV LTA was produced in an *Escherichia coli* based expression plasmid. ATPase activity was measured using the malachite green assay. A high throughput screen was completed using a brain-biased library of 75,000 drug-like compounds selected for physicochemical properties consistent with blood brain barrier permeability.

Results—Five compounds showed non-competitive inhibition of ATPase activity with an EC₅₀ 15 μM. Modest antiviral activity was demonstrated in an immunofluorescence assay for JCV VP-1 expression in COS7 cells (EC₅₀ 15, 18, 20, 27, and 52 μM respectively). The compounds also inhibited viral replication in a real time PCR assay at comparable concentrations. LD₅₀ in the MTS96 and Cell TiterGlo assays was >100 μM for all compounds in COS7 as well as HEK293 cells. However, two compounds inhibited cell proliferation in culture with IC₅₀ values of 43 and 34 μM respectively. Despite substantial amino acid similarity between polyomavirus JC, BK and SV40 proteins, these compounds differ from those previously reported to inhibit SV40 LTA ATPase in chemical structure as well as a non-competitive mechanism of inhibition.

Conclusion—LTA ATPase is a valid target for discovery. Additional screening and chemical optimization is needed to develop clinically useful compounds with less toxicity, which should be measured by metabolic as well as cell proliferation assays.

Keywords

Polyomavirus JC; Large T antigen; ATPase; Drugs

1. Introduction

Polyomavirus virus JC (JCV) is widely prevalent in man (Major and Curfman, 1997). It is believed that following primary infection in childhood, the virus becomes latent in the kidney, B-lymphocytes, and possibly the brain (Gallia et al., 1997; Lafon et al., 1998; Monaco et al., 1996). JCV is best known as the etiologic agent of progressive multifocal encephalopathy (PML) in patients with Acquired Immune Deficiency Syndrome (AIDS). PML has received a lot of recent attention due to its occurrence in patients with multiple sclerosis treated with the immunomodulating monoclonal antibodies (Kleinschmidt-DeMasters and Tyler, 2005; Langer-Gould et al., 2005), and in immune deficiency states (Freim Wahl et al., 2007; Kranick et al., 2007; Weber et al., 2001). PML is a destructive infection of oligodendrocytes in the white matter, and leads to neurologic deficits corresponding to lesions in the cerebral hemispheres, cerebellum, or brainstem. The prognosis of PML in AIDS patients has improved with highly active anti-retroviral treatment (HAART), but remains difficult to predict, and overall one-year survival is only 50% (Clifford et al., 1999). No proven anti-viral treatment is available for PML at this time. We hypothesize that JCV inhibitory drugs can be discovered by screening chemical libraries for compounds that can inhibit the ATPase machinery associated with JCV large T antigen (LTA). LTA is good target for drug discovery because (a) it is a key viral protein required for DNA replication, (b) it is well conserved across multiple viral strains, and (c) there is no homologous protein present in human cells, which offers of the prospect of developing anti-viral compounds with an acceptable toxicity profile. LTA directs the initiation of viral DNA replication by assembly into a double hexameric helicase which unwinds the duplex DNA bidirectionally (Gai et al., 2004). These biochemical changes are energy dependent, and an ATPase domain as well as an ATP binding site are present in the LTA protein. Hence, it is reasonable to expect that small molecule inhibitors of LTA ATPase will be detrimental to viral replication. Accordingly, this study presents the results of a high throughput screening campaign to discover anti-JCV compounds using an ATPase assay.

2. Materials and methods

A brief outline of the experimental procedures used follows. Greater details are provided in a supplemental data file.

2.1. Production of recombinant JCV LTA

JCV LTA was produced from a glutathione *S*-transferase (GST) fusion protein of the intronless JCV large T antigen (pGEX1-LTA). The kinetic parameters of LTA ATPase enzyme activity were determined using malachite green detection of free phosphate generated by the reaction. Determination of the K_m for ATP was based on the Michaelis–Menten equation.

2.2. High throughput screening (HTS) for inhibitors of ATPase activity

An in-house chemical library consisting of about 75,000 compounds was used in a 384-well plate format high-throughput screen assay to identify inhibitors of JCV ATPase. Primary screening hits were defined as compounds that inhibited 70% ATPase activity. Hits were

retested from the compound library in 5 point dose response experiments using the same reaction conditions to confirm inhibition.

2.3. Hit validation and characterization

Selected hit compounds were tested in 12-point dose response from fresh powder using the same assay conditions as the screen. ATPase IC₅₀ values were calculated using a sigmoidal dose response equation. The mechanism of inhibition was determined using the method of replots for a single substrate enzyme reaction. Data were fit to the Michaelis–Menten equation to determine $V_{\max}$ and K_m for each concentration of inhibitor.

2.4. Q-PCR assay for measuring JCV replication

Real time PCR was used to directly measure JCV replication in Cos-7 cells. Simultaneous quantitation of a housekeeping gene ribosomal protein 32 (RPL32) sequence allowed monitoring of host cell replication (Ahn et al., 2008). A 50% JCV inhibitory drug concentration (PCR IC₅₀), and a 50% lethal (toxic) concentration (PCR LD₅₀) were determined.

2.5. Fluorescent focus assays for measuring JCV VP-1 gene expression

Seventy percent confluent Cos-7 cells in tissue culture plates with coverslips were infected with JCV Mad-4 stock. Quantitation of the percent of cells expressing VP-1 antigens was performed using whole slide image analysis after staining with a rabbit polyclonal antibody to SV40 VP-1 (Abcam 53977). Drug concentrations effecting 50% reduction in VP-1 positive cells and total cell nuclei were respectively used to calculate VP-1 IC₅₀ and VP-1 LD₅₀.

2.6. Cell viability assays

To determine possible cytotoxicity of the compounds, three methods for evaluating cell viability were used according to the manufacturer's protocols: the CellTiter 96 AQueous MTS assay, the CellTiter-Glo[®] Luminescent Cell Viability Assay, and cell counts were obtained using the Celigo Adherent Cell Cytometer. HEK293 or COS7 cells were plated at 3000 cells/well in 384-well plates and treated with compounds concentrations ranging from 100 μ M to 45 nM to generate 12-point dose response curves for calculation of LD₅₀. Cells were incubated with compound for 24 h, 48 h, or 6 days prior to addition of detection reagent or reading on cytometer.

3. Results

3.1. Development of ATPase assay for high-throughput screening

Initial assay development involved characterizing the kinetics of the signal generated by LTA mediated hydrolysis of ATP, with the released inorganic phosphate binding to malachite green. Using 50 nM enzyme, the reaction was linear up to 3 h (Fig. 1). Next, the K_m for ATP was determined to be 73.4 ± 4.8 μ M (Fig. 2). Therefore, LTA concentration of 50 nM and ATP concentration of 75 μ M were selected for screening to obtain hits with a competitive, non-competitive or un-competitive mechanism of action. Individual buffer components were optimized (data not shown). Manganese had a much lower $V_{\max}$ and

higher K_m than magnesium favoring magnesium as the metal to complex with ATP. The pH optimum was determined to be pH 7 with higher pH buffers reducing enzyme activity (V_{max}/K_m) by 50%. The final concentration of DMSO in the ATPase enzyme activity assay was 1%, although it was determined that the tolerated up to 10% DMSO concentration.

3.2. High-throughput screen

A threshold of 70% inhibition was used to identify 93 hits from the screened library, a hit rate of ~0.1%. Representative data is shown in Fig. 3. Z' factors were used to characterize the quality of data on each assay plate. The average Z' factor for the screen was 0.77 ± 0.09 (Fig. 3C). Of the 225 plates tested in the screening campaign, 12 had calculated Z' factors less than 0.6. Each of these plates was retested and yielded improved Z' factors. The chemical structures of these compounds were reviewed, and 78 selected for validation from the compound library stocks. Each of these compounds was retested in a 5 point dose response experiment. Of the 78 compounds tested, 55 compounds demonstrated reproducible, dose dependent inhibition of JCV ATPase activity. Fifteen of these compounds representing different structural classes and a range of dose response profiles were selected for further characterization. Fresh powder of the compound was obtained and tested in a twelve-point dose response experiment to accurately determine potency. Representative data on ATPase inhibition is shown in Fig. 4 and Table 1.

3.3. Characterization of selected screening hits

Compounds with IC_{50} s in the ATPase assay $<20 \mu M$ were further characterized to determine mechanism of inhibition and evaluate antiviral activity. Inhibitors were cross titrated versus ATP, and dependence of V_{max} and V_{max}/K_m plotted (representative data, Fig. 5). The mechanism of inhibition was determined based on the dependence of V_{max} and V_{max}/K_m on inhibitor concentration. If both V_{max} and V_{max}/K_m are dependent on inhibitor concentration, as is the case with LDN-0015182 (Fig. 5) and the compounds listed in Table 1, this suggests a non-competitive mechanism of inhibition in which the inhibitor can bind to either the free enzyme or the enzyme substrate complex. Allosteric inhibition increases the probability that the compounds will be selective for the target of interest rather than pan-ATPase inhibitors. Therefore, compounds exhibiting non-competitive inhibition were further evaluated for antiviral activity.

Modest antiviral activity was demonstrated in an immunofluorescence assay for JCV VP-1 expression in COS7 cells (VP-1 IC_{50} 18.1 ± 8.2 , 15.1 ± 4.1 , 52.5 ± 17.7 , 19.9 ± 6.2 , $26.6 \pm 16.3 \mu M$ respectively, expressed as mean $\pm$ SD) (Table 1). Representative experiments for LDN 0015182 are presented in Figs. 6 and 7. In quantitative real time PCR based assays designed to directly measure viral replication in COS7 and POJ cells, LDN-0063710 and LDN-0065780 showed no activity at concentrations up to $100 \mu M$, while the remaining three compounds had an EC_{50} between 50 and $100 \mu M$. LDN-0012753 and LDN-0015182 inhibited cell proliferation with LD_{50} values of 42.9 ± 2.6 and $34.2 \pm 8.2 \mu M$ respectively, while the remaining three compounds did not inhibit cell proliferation at the highest concentration tested. In viability assays not dependent on cell proliferation, namely the MTS96 aqueous and Cell TiterGlo assays, the LD_{50} was $>100 \mu M$ for all compounds in COS7 as well as HEK293 cells. Chemical structures of the compounds are provide in Fig. 8.

4. Discussion

The experiments performed show that that LTA-ATPase inhibition by small molecules has a detrimental effect on the viral life cycle. Five compounds exhibiting non-ATP-competitive inhibition of JCV ATPase activity that were identified in the initial high throughput screen also inhibited JCV VP-1 expression in COS7 cells. Since COS7 cells are transformed with SV40 the inhibitory effect of the aforementioned compounds may be partly mediated by an effect on SV40 T-antigen ATPase activity.

The cytotoxicity of these compounds was assay dependent. No toxicity at 100 μ M was observed in the Cell TiterGlo™ assay which assesses cell viability based on intra-cellular ATP content. Toxicity was also not observed in the MTS assay which relies on the presence of dehydrogenase enzymes found in metabolically active cells. In contrast, the compounds appeared toxic in a fluorescent focus assay in which we saw a reduction in the number of DAPI stained nuclei following drug treatment of the cultured cells. This effect was confirmed in a PCR assay that directly measured cell proliferation by quantitation of DNA copy numbers for a house-keeping gene (ribosomal protein 32). It is possible that with chemical optimization of the hits to improve potency, there may be a clearer separation between activity and cytotoxicity.

Animal and human studies are needed to clarify the implications of these divergent cytotoxicity evaluations. Human PML lesions typically do not contain actively replicating oligodendroglial cells. Indeed these cells progressively decrease in number as the disease progresses. Therefore, one could argue that the anti-proliferative cytotoxic effects observed should not discourage us from pursuing the further development of this class of compounds. Nevertheless, assessment of toxicity in mitotically active organs (liver, gastrointestinal tract, bone marrow) make it very desirable that future investigations specifically include a cell proliferation assay in the compound evaluation process. This is frequently not been done as can be illustrated by publications describing an anti-polyomavirus action for 5 HT1 receptor antagonists (Elphick et al., 2004), ricin (Nelson et al., 2013), and Mefloquine (Brickelmaier et al., 2009). It is notable that a clinical trial of Mefloquine in patients with PML had to be prematurely terminated due to lack of efficacy (Clifford et al., 2013).

While this is the first study that has focused on JCV, the LTA ATPase domain has been explored as a drug discovery target for two other polyomaviruses, namely BK and SV40 (Seguin et al., 2012a). The latter screening effort resulted in identification of a class of Bisphenols which inhibited SV40 large T antigen ATPase activity with EC₅₀s ranging from 7 to 26 μ M. Lineweaver–Burk plots suggested that the mechanism of action was competition with ADP for the active site on the enzyme. These compounds were more cytotoxic than those described in our study: Cell-TiterGlo viability for the compounds reported was noted to fall sharply after 12.5 μ M with no live cells remaining at 100 μ M. In a follow up publication this research group describes screening of an in-house library of 150 dihydropyrimidine analogs and 2240 FDA approved compounds belonging to the MicroSource MS2000 collection. Activity against both polyomavirus BK and SV40 was evaluated using recombinant SV40 protein. SV40 LTA ATPase inhibition >20–35% was observed in 46 compounds that included flavones, polyphenols and bisphenols, but only two

compounds, bithionol and hexachlorophene, were evaluated in detail (Seguin et al., 2012b). Both compounds were more cytotoxic than our LDN series of compounds: the MTS assay generated IC₅₀ values of 68 and 55 μ M respectively. Although therapeutic indices of 31 and 17 respectively were quoted based on in vitro ATPase inhibition, quantitative PCR assay showed only about ~70% inhibition of BKV and SV40 DNA replication at 30 μ M, indicating a much lower selectivity of anti-viral activity at the cellular level. The toxicity of these compounds was not examined in cell proliferation assays.

Our screen differs from those described above in having used recombinant JCV rather than SV40 LTA in a search for ATPase inhibitors. Overall, there is 71% amino acid identity and 17% amino acid similarity between the two proteins. The Walker A motif (P-loop) and other critical arginine and lysine residues that specifically interact with ATP are also conserved. Nevertheless, the JCV protein has 20 fewer amino acids (708 versus 688) and 12% of the amino acids are dissimilar. Five of the dissimilar residues are located on the ATP binding domain, and one of these differences results in a non-polar amino acid (alanine) being replaced by a polar moiety (serine) (Topalis et al., 2013). There are also differences in splicing pattern for these two proteins: the spliced variants result in truncated proteins that can interact with pRB, p107, and p300 (but not with the helicase/ATP domain), and trigger entry of the host cell into the G1/S phase. It is worth noting that whereas the inhibition of the JCV encoded enzyme by the LDN series of compounds is non-competitive, bithionol and hexachlorophene act on the corresponding SV40 protein by a competitive mechanism. Crystallographic studies of the VP-1 protein show significant differences in how even closely related polyomaviruses interact with their respective cellular receptors (Neu et al., 2010).

LTA Domains other than the ATPase domains have also received limited scrutiny as targets for drug discovery. A screen of the NCI Diversity set of compounds led to identification of a compound 'B11' that inhibited the binding of SV40 LTA to cellular p53 in an ELISA assay (Carbone et al., 2003). Another approach tried to leverage the ability of SV40 LTA J-domain to bind the Hsp70 ATPase domain, which has been reported to be essential for viral replication (Sullivan and Pipas, 2002). Hsp70 is endowed with cell growth promoting and anti-apoptotic actions. Therefore, inhibitors of Hsp70 would be expected to curtail cell growth as well as polyomavirus replication by creating a less favorable milieu for the virus to thrive, and perhaps even causing selective apoptosis of infected cells (Wright et al., 2008). Moreover, Hsp70 and the cochaperone Hsp40 have been implicated in the correct folding of the SV40 VP-1 capsid protein into functional pentamers (Li et al., 2009; Watanabe et al., 2013). Accordingly, an Hsp70 inhibitor, MAL2-11B, has been shown to cause one log reduction of BKV viral load in HK2 cells at concentrations of 15 μ M, and a fivefold reduction in SV40 DNA replication at a concentration of 100 μ M (Wright et al., 2009). Unfortunately, this compound is even more toxic than the ATPase inhibitor Bisphenol A.

In conclusion, we provide proof of principle that the LTA ATPase is a valid target for discovery of anti-JCV drugs. The hits identified in this campaign are reasonable starting points for medicinal chemistry to improve potency and selectivity. Screening of additional chemical libraries could also be considered since the potential market for anti-JCV drugs is

considerable, particularly if we can develop safe compounds for prophylactic use. The total number of HIV infections in the USA is estimated to be 1.2 million, with 56,000 new cases occurring annually (Hall et al., 2008).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BKV	polyomavirus BK
JCV	polyomavirus JC
LTA	large T antigen
PML	progressive multifocal encephalopathy

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.antiviral.2014.10.004>.

Highlights

- Polyomavirus JC large T antigen is a valid target for drug development as this protein plays a critical role in viral replication.
- Recombinant JCV LTA was produced and utilized in an ATPase activity based high throughput screen of 75,000 drug-like compounds selected for predicted blood brain barrier permeability.
- Five compounds showed non-competitive inhibition of ATPase activity with an EC_{50} 15 μ M. Modest antiviral activity was demonstrated but additional screening and chemical optimization is needed to develop clinically useful compounds.

Fig. 1. Time course of inorganic phosphate release at different concentrations of JCV ATPase enzyme using malachite green detection of phosphate release in the presence of 100 μ M ATP. The X-axis depicts time in minutes. The Y-axis plots the nmol free PO_4 detected by malachite green.

Fig. 2.

Initial velocity measurements were used to determine the K_m for ATP. This determination was performed using 50 nM JCV ATPase in a 50 μl reaction volume with varying concentrations of ATP. The reaction was stopped at 60 min by addition of 20 μl malachite green detection, developed for 60 min, then absorbance measured at 620 nm. Absorbance was converted to nmol PO_4 . K_m value for ATP was calculated to be $73.4 \pm 4.8 \mu\text{M}$ based on Michaelis–Menten equation. The data shown is a representative graph from 5 experiments.

Fig. 3.

Representative data from high-throughput screen. (A) Data from a single 384-well plate. Columns 1–22 of the compound plate contain test compounds, and column 23 and 24 are DMSO controls. Column 23 contains ATPase + substrate (circled in black) and column 24 contains substrate only (circled in green). (B) Data from a screening run consisting of 21 plates. Compounds with negative inhibition are presumably activators of ATPase activity. (C) Calculated Z prime values for the entire set of 225 plates screened. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Determination of potency for selected hit compounds. Compounds were tested in 12 point-dose response assays using JCVirus ATPase activity assay to determine IC_{50} values. X-axis is the compound concentration and the Y-axis is the percent inhibition normalized to controls. Data is representative of at least 2 independent tests.

Fig. 5.

Evaluation of mechanism of inhibition of LDN-0015182. Initial velocities of the JCV ATPase were plotted versus ATP concentration in the presence of increasing concentration of inhibitor (A) to determine k_{cat} and K_{cat}/K_m at each inhibitor concentration. Dependence of k_{cat} (B) and K_{cat}/K_m (C) on inhibitor concentration was used to determine mechanism of inhibition. Results are representative of 3 independent experiments.

Fig. 6. Inhibition of anti-viral activity in COS7 cells using the fluorescent assay. Effect of LDN-0015182 on JCV VP-1 expression expressed as a percentage of expression seen in cells not treated with drug ($n = 4$ independent experiments). X-axis is the compound concentration and Y-axis is the percent inhibition. Error bars smaller than the width of the line graph are not visible.

Fig. 7. JCV VP-1 expression in Cos7 cells with (right panels) or without (left panels) exposure to 30 μ M LDN 0015182. Image analysis was done to quantify the percentage inhibition of signal captured from the entire cover slip (upper panels). Higher magnification images (lower panels) illustrate that VP-1 staining had an exclusively nuclear localization.

LDDN	Structure
LDN-0012753
LDN-0015182
LDN-0060230
LDN-0063710
LDN-0065780

Fig. 8.
Chemical structures of the compounds with anti-JCV activity identified from the high throughput screen.

Table 1

Antiviral activity and cytotoxicity of selected compounds.

Compound	IC ₅₀ ATPase (μM) ± SD	IC ₅₀ VP1 (μM)	LD ₅₀ VP-1 (μM)	LD ₅₀ Cell TiterGlo	LD ₅₀ MTS assay
LDN-0012753*	0.41 ± 0.34	18.1 ± 8.2	42.9 ± 2.6	>100	>100
LDN-0015182*	.73 ± 0.3	15.1 ± 4.1	34.2 ± 8.2	>100	>100
LDN-0060230*	12.5 ± 3.6	19.9 ± 6.2	>50	>100	>100
LDN-0063710	3.6 ± 0.14	52.5 ± 17.7	>50	>100	>100
LDN-0065780	3.4 ± 1.34	26.6 ± 16.3	>50	>100	>100

* EC₅₀ measured by quantitative PCR was between 50 and 100 μM for the indicated compounds. For the remaining two compounds it was >100 μM. Higher concentrations could not be tested due to considerations of solubility.